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### CARBOXYPEPTIDASE A, EVIDENCE FOR AN ANHYDRIDE INTERMEDIATE

In the reaction of carboxypeptidase A, a  $\text{Zn}^{2+}$  containing exopeptidase, it is still in discussion, whether Glu-270 forms a covalent anhydride intermediate with the acyl group of the substrate or is involved in a general base catalysis mechanism. Evidence for an acyl enzyme has been obtained at  $-60^\circ\text{C}$  only spectroscopically with one ester substrate, the *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactate (Makinen).

Since it should be possible to trap such an anhydride intermediate with nucleophiles under conditions where accumulation can be expected, we incubated the enzyme at  $-75^\circ\text{C}$  with several hippuric esters or -peptides in the presence of hydroxylamine and *O*-methylhydroxylamine. Thus the enzyme could be inactivated, while it was still active in the blank probe with the same concentration of hydroxylamine. Inactivation was most efficient with hippuryl glycolic acid, a relatively slow substrate for the carboxypeptidase A. A hydroxamate group within the protein could be identified by the visible spectrum of its  $\text{Fe}^{3+}$ -complex, which was characteristic for that of a glutamic acid. Such a hydroxamate group, responsible for the inactivity of the enzyme and produced only in the presence of a substrate, can be expected, if this glutamic acid forms an anhydride intermediate. Furthermore we could stabilize the acyl enzyme by adding denaturing agents at  $-60^\circ\text{C}$  during the reaction with hippuryl-L-phenylalanine or *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactate. This loaded enzyme was reduced with  $[\text{H}]\text{NaCNBH}_3$ . After complete hydrolysis of the protein the  $[\text{H}]$   $\alpha$ -ami-

no- $\delta$ -hydroxyvaleric acid, which is formed as the reduction product of an acylated glutamic acid, could be identified by high voltage paper electrophoresis and paper chromatography. These results can also be interpreted only on the basis of an anhydride intermediate.

A tryptic digest of the reduced protein gives evidence that the glutamic acid in question is Glu-270.



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### THE INTERACTION OF ZINC IONS WITH ARSANILAZOTYROSINE-248-CARBOXYPEPTIDASE A

Arsanilazotyrosine-248-carboxypeptidase A([(Azo-CPD)Zn]) [1] is a derivative modified with chromophoric arsanilazotyrosine-248 residue. [(Azo-CPD)Zn] has almost the same activity as native carboxypeptidase A. It has proven particularly useful for studying the conformation of the enzyme [2] and metal binding to the apo-enzyme [3]. At pH 7.5-8.8, the arsanilazotyrosine-248 residue forms an intramolecular complex with the zinc ion at the active site and gives a characteristic optical absorption at 510 nm.

When excess zinc ions were added to [(Azo-CPD)Zn], the characteristic red color, which arose from the intramolecular complex of the arsanilazotyrosine-248 residue with the active

site zinc of the enzyme, changed to yellow (Fig. 1) Excess zinc ions also inhibited the peptidase activity of [(Azo-CPD)Zn]. The interaction between

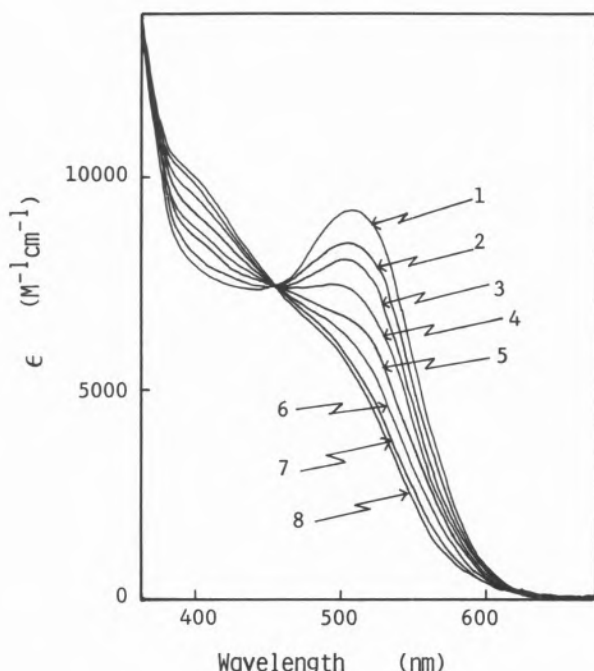
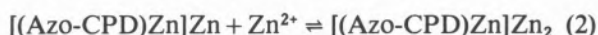
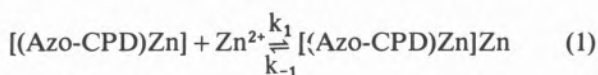


Fig. 1

Effect of excess zinc ions on the absorption spectrum of arsanilazotyrosine-248-carboxypeptidase A. [(Azo-CPD)Zn] =  $3.4 \times 10^{-5}$  M, Temp 25°C. pH 8.2, 0.05 M Tris-HCl buffer (0.5 M NaCl).

1, without zinc ions; 2,  $Zn^{2+} = 10^{-5}$  M; 3,  $Zn^{2+} = 2.0 \times 10^{-5}$  M; 4,  $Zn^{2+} = 3.0 \times 10^{-5}$  M; 5,  $Zn^{2+} = 5.0 \times 10^{-5}$  M; 6,  $Zn^{2+} = 7.0 \times 10^{-5}$  M; 7,  $Zn^{2+} = 1.5 \times 10^{-4}$  M; 8,  $Zn^{2+} = 3.5 \times 10^{-4}$  M

excess zinc ions and [(Azo-CPD)Zn] has been studied by the stopped flow and spectrophotometric methods at pH 8.2, 7.7, I=0.5M (NaCl) and 25°C. [(Azo-CPD)Zn] has two binding sites for excess zinc ions and the binding constant of the first site ( $3.9 \times 10^5$  M<sup>-1</sup> at pH 8.2,  $7.1 \times 10^4$  M<sup>-1</sup> at pH 7.7) is much larger than that of the second site ( $1.8 \times 10^3$  M<sup>-1</sup> at pH 8.2,  $7 \times 10^2$  M<sup>-1</sup> at pH 7.7), as shown in the following equations.



The binding of excess zinc ions to the first site was completely correlated with both the inhibition of the peptidase activity and the color change of

the enzyme. The results can be explained in terms of the zinc ion reaction with only one of three conformational states of [(Azo-CPD)Zn] [2]. The second order rate constants ( $k_1$ ) for binding of excess zinc ions to [(Azo-CPD)Zn] were  $4.3 \times 10^6$  and  $8.4 \times 10^5$  M<sup>-1</sup>sec<sup>-1</sup>, at pH 8.2 and 7.7, respectively, and the first order rate constants ( $k_{-1}$ ) for the dissociation of zinc ions from [(Azo-CPD)Zn]<sub>2</sub> are 11 and 12 sec<sup>-1</sup>, respectively. It has been proven that excess zinc ions promote the inhibition of the peptidase activity and the color change from red to yellow through specific binding of zinc ions to one conformational state of [(Azo-CPD)Zn].

## REFERENCES

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PS2.28 — TH

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## KINETIC SALT EFFECT OF ALKALINE EARTH CHLORIDES ON THE UREASE CATALYZED UREA HYDROLYSIS

The kinetics of the Ni-containing [1] enzyme urease have turned out to be very complex. In order to get some informations about the reaction mechanism of the urease catalyzed urea hydrolysis we investigated the reaction in presence of the alkaline earth chlorides MgCl<sub>2</sub>, CaCl<sub>2</sub>, SrCl<sub>2</sub>, and BaCl<sub>2</sub> in a concentration range of 10<sup>-6</sup>